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(71) Applicant: VALENTIS, INC. [US/US]; 863A Mitten Road, Burlingame, CA 94010 (US).

(72) Inventors: WANG, Jinkang; 39 Inverness Drive, San Francisco, CA 94132 (US). ZHANG, YiLin; 444 N. El Camino Real #303, San Mateo, CA 94401 (US). WEDEKING, Todd; 2961 Fleetwood Drive, San Bruno, CA 94066 (US).

(74) Agents: BASTIAN, Kevin, L. et al.; Townsend and Townsend and Crew LLP, 8th floor, Two Embarcadero Center, San Francisco, CA 94111 (US).

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(54) Title: METHODS AND COMPOSITIONS FOR DELIVERING NUCLEIC ACIDS

(57) Abstract

The invention provides cationic amino sugar derivatives useful in delivering nucleic acids by intramuscular, subcutaneous or intradermal injection. The cationic amino sugar derivative is formulated with the nucleic acid to be delivered in a transfection complex.

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METHODS AND COMPOSITIONS FOR DELIVERING NUCLEIC ACIDS

Field of the Invention

This invention relates to carriers for delivering nucleic acids to cells. In particular, the invention relates to new cationic amino sugar derivative molecules suitable for delivering nucleic acids to a mammal.

Background of the Invention

The introduction of genetic material into a cell can facilitate expression of an encoded protein to induce an immune response or complement a deficient or defective protein. The use of such technology allows for the treatment of disease as well as production of certain proteins in an *in vitro* application.

One method of introducing nucleic acids into a cell is mechanically, using direct microinjection. However this method is labor-intensive and, therefore, only practical for transfecting small numbers of cells such as eukaryotic germline cells for the production of transgenic systems. To be effective in treating a disease, a nucleic acid-based therapy typically must enter many cells.

Gene transfer entails distributing nucleic acids to target cells and then transferring the nucleic acid across a target cell membrane intact and, typically, into the nucleus in a form that can function in a therapeutic manner. *In vivo* gene transfer is complicated by serum interactions, immune clearance, toxicity and biodistribution, depending on the route of administration.

Many *in vivo* gene transfer methods under study in the clinic use viral vectors. Although viral vectors have the inherent ability to transport nucleic acids across cell membranes and some can integrate exogenous DNA into the chromosomes, they can carry only limited amounts of DNA and also pose risks. One such risk involves the random integration of viral genetic sequences into patient chromosomes, potentially damaging the genome and possibly inducing a malignant transformation. Another risk is

that the viral vector may revert to a pathogenic genotype either through mutation or genetic exchange with a wild-type virus.

More recently, cationic lipids have been used to deliver nucleic acids to cells, allowing uptake and expression of foreign genes both *in vivo* and *in vitro*. When used for *in vivo* delivery, the role of the cationic lipid carriers is further complicated by the interactions between the lipid-nucleic acid complexes and host factors, e.g., the effects of the lipids on binding of blood proteins, clearance and/or destabilization of the complexes.

Typically, cationic lipids are mixed with a non-cationic lipid, usually a neutral lipid, and allowed to form stable liposomes, which liposomes are then mixed with the nucleic acid to be delivered. The liposomes are mixed with nucleic acid in solution, at concentrations and ratios optimized for the target cells to be transfected, to form cationic lipid-nucleic acid transfection complexes. Another method of complex formation involves the formation of DNA complexes with mono- or poly-cationic lipids without the presence of a neutral lipid. While the use of cationic lipid carriers for transfection is now well established, structure activity relationships are not well understood. It is postulated that different lipid carriers will affect each of the various steps in the transfection process (e.g., condensation, uptake, nuclease protection, endosomal release, nuclear trafficking, and decondensation) with greater or lesser efficiency, thereby making the overall transfection rate difficult to correlate with lipid structures. Thus, alterations in either the cationic or neutral lipid component do not have easily predictable effects on activity

Despite the advances obtained with the cationic lipids, there exists a need for alternative carriers to deliver nucleic acids to cells. The alternative carriers should be suitable for formulation into active transfection compositions. The present invention addresses these and other needs.

Relevant Literature

Transfection of mouse muscle cells in vivo with a reporter gene is describe in Wolfe, J.A., et al., (1990) Science 247:1465-1468, Acsadi G., et al., (1991) Nature 352:815-818, and Wolfe, J.A., et al., (1991) BioTechniques 11(4):474-485. The use of gene transfer for vaccination purposes is described in Felgner, P.L. and G. Rhodes (1991)

Nature 349:351-352 and PCT/US90/01515; Fazio V.M. (1997) Res. Virol. 148:101-108; Levitsky H.I. (1997) Nat. Biotechnol. 15:619-620; Conry R.M., et al., (1996) Gene Ther. 3:67-74; Conry R.M., et al., (1996) Semin. Oncol. 23:135-147; Haynes, J.R., et al., (1996) Adv. Drug Deliv. Rev. 21:3-18; and Ulmer, J.B., et al., (1996) Adv. Exp. Med Biol. 397:49-53.

The use of cationic peptides and proteins in DNA delivery is described in Emi et al., (1997) Biochem Biophys Res. Comm. 231(2):421-424 (polyarginine); Fritz et al., (1996) Hum. Gene Ther. 7(12):1395-1404 (histone H1 and SV40 large T antigen nuclear localizing signal); Gao and Huang (1996) Biochemistry 35(3) 1027-1036 (poly-L-lysine, protamine); Legendre and Szoka (1993) Proc. Natl. Acad. Sci USA 90(3):893-897 (gramicidin S); and Niidome et al., (1997) J. Biol. Chem. 272(24):15307-15312 (cationic alpha-helical oligopeptides). Additional transfection facilitating agents are described in Ibanez, et al., (1996) Biochem Cell Biol 74(5):633-643 (spermidine); Budker et al., (1997) Biotechniques 23(1):139 (histone H1 and amphipathic polyamines); and Barthel et al., (1993) DNA Cell Biol. 12(6):553-560 (lipospermine).

The use of poly-L-lysine conjugated to specific receptor ligands for DNA delivery is described in Wu, G.Y. and Wu, C.H. (1988) J. Biol Chem. 263:14621-14624. Targeting with polylysine conjugated to receptor ligands or to antibodies is reviewed in Deshmukh & Huang (1997) New J. Chem. 21:113-124. Glycosylated poly-L-lysine for DNA delivery is described in Proc. Natl. Acad. Sci. USA 91:4086 (1997). The use of gluconoylated polylysine for DNA delivery is described in U.S. Patent No. 5,595,897.

The use of polyvinylpyrolidone for DNA delivery via IM injection is described in Mumper, R.J., et al., (1996) Pharm Res. 13:701-709 and WO 96/21470.

Methods for enhancing transient gene expression in eukaryotic cells using amino acid and carboxylic derivatives are described in PCT/US97/19860.

Anti-tumor and interferon producing compositions of Tobramycin complexed with double-stranded RNA are described in U.S. Patent No. 4,400,375.

Summary of the Invention

The invention provides compositions for improved delivery and expression of genes. In one embodiment, the invention provides a method of introducing a DNA

molecule into a cell of a mammal, the method comprising contacting a cell with a nucleic acid/cationic amino sugar derivative complex.

The cationic amino sugar derivative can be an aminoglycoside; an antibiotic; a monosaccharide, such as amiprilose or streptidine; a disaccharide, such as, fortimicins, neamine, sisomicin, or streptobiosamine; a trisaccharide, such as amikacin, arbekacin, deoxydihydrostreptomycin, destomycin A, dibekacin, dihydrostreptomycin, genticin, gentamicin, hygromycin B, isepamycin, kanamycin A, kanamycin B, kanamycin C, micronomicin, paromomycin, ribostamycin, streptomycin, streptonicozid, or tobramycin; or a spermine moiety.

The complex can be delivered intramuscularly, intradermally, intraperitoneally, intratracheally, or intra-articularly.

In one embodiment, the DNA molecule comprises an expression cassette, that can encode a therapeutic protein or a protein that induces an immune response in a mammal.

In another embodiment, the invention provides a pharmaceutical composition comprising a pharmaceutically acceptable excipient and a complex comprising the nucleic acid molecule and a cationic amino sugar derivative.

The cationic amino sugar derivative of the pharmaceutical composition can be an aminoglycoside; an antibiotic; a monosaccharide, such as amiprilose or streptidine; a disaccharide, such as, fortimicins, neamine, sisomicin, or streptobiosamine; a trisaccharide, such as amikacin, arbekacin, deoxydihydrostreptomycin, destomycin A, dibekacin, dihydrostreptomycin, genticin, gentamicin, hygromycin B, isepamycin, kanamycin A, kanamycin B, kanamycin C, micronomicin, paromomycin, ribostamycin, streptomycin, streptonicozid, or tobramycin; or a spermine moiety.

In the pharmaceutical composition, the DNA molecule can be an expression cassette that can encode a therapeutic protein or a protein that induces a protective immune response in the mammal.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. For purposes of the present invention, the following terms are defined below.

The term "cationic amino sugar derivative" refers to a derivative of a sugar molecule that contains a cationic group comprising nitrogen. Such derivatives have a cationic domain that is capable of complexing with DNA and a neutral hydrophilic domain (the sugar moiety).

The sugar molecule can be monomeric or oligomeric, e.g., disaccharide, trisaccharide, tetrasaccharide or pentasaccharide. The simplest sugars are comprised of monosaccharide units, which are aldehydes or ketones with two or more hydroxyl groups. They typically have 3-7 carbons, usually 5 or 6 carbons forming furanose and pyranose rings. Monosaccharide units are linked to each other by glycosidic bonds to form disaccharides and other polysaccharides. Additionally, sugars may be in the form of aminoglycosides when the anomeric carbon is linked to the nitrogen atom of an amine, forming a glycosidic bond. In the present invention, an aminoglycoside is typically an antibiotic.

The sugar moiety may also include modifications well known to those skilled in the art. For instance one or more hydroxyl groups may be replaced with halogens, alkyl groups, amines, azido groups or functionalized as ethers or esters. Additionally, the entire sugar may be replaced with sterically and electronically similar structures, including aza-sugars and carbocyclic sugar analogs.

The cationic domain of the cationic amino sugar derivative contains at least one nitrogen atom. The cationic domains include but are not limited to protonated amines, quaternary amines, guanidines, spermine, spermidine, putrescine and other polyamines capable of condensing DNA.

"Nucleic acid molecule", as used herein, may be DNA, RNA, an oligonucleotide, or a plasmid containing an expression cassette. Nucleic acids may also include modified nucleotides, including those that increase stability and those that permit correct read through by a polymerase and those that do not alter expression of a polypeptide encoded by that nucleic acid.

"Cationic amino sugar derivative-nucleic acid complex" refers to a combination of a cationic amino sugar derivative and a nucleic acid for use in transfecting cells.

The phrase "expression cassette", refers to nucleotide sequences which are capable of affecting expression of a structural gene in hosts compatible with such sequences. Such cassettes include at least promoters and optionally, transcription termination signals. Additional factors necessary or helpful in effecting expression may also be used as described herein.

The term "operably linked" when used in reference to a promoter and a DNA coding sequence refers to linkage of a promoter upstream from a DNA sequence such that the promoter mediates transcription of the DNA sequence.

The term "recombinant" refers to DNA that has been isolated from its native or endogenous source and modified either chemically or enzymatically to delete naturally-occurring flanking nucleotides or provide flanking nucleotides that do not naturally occur. Flanking nucleotides are those nucleotides which are either upstream or downstream from the described sequence or sub-sequence of nucleotides.

The term "effective amount" is intended to mean the amount of vector or drug which achieves a positive outcome on on the disease indication or test system for which it is being used.

The term "contacting a cell" when referring to contacting with a cationic amino sugar derivative-nucleic acid complex is used herein to refer to contacting in a manner such that the cationic amino sugar derivative- nucleic acid complex is internalized into the cell. In this context, contacting a cell with a cationic amino sugar derivative-nucleic acid complex is equivalent to transfecting a cell with a nucleic acid *in vivo* or *in vitro*.

"Transfection" as used herein means the delivery of exogenous nucleic acid molecules to a cell, either *in vivo* or *in vitro*, whereby the nucleic acid is taken up by the cell and is functional within the cell. A cell that has taken up the exogenous nucleic acid is referred to as a "host cell" "target cell" or "transfected cell." A nucleic acid is functional within a host cell when it is capable of functioning as intended. Usually, the exogenous nucleic acid will comprise an expression cassette which includes DNA coding

for a gene of interest, with appropriate regulatory elements, which will have the intended function if the DNA is transcribed and translated, thereby causing the host cell to produce the peptide or protein encoded therein.

Brief Description of the Drawings

Figure 1 provides a schematic of the reaction to produce a compound of the invention.

Figure 2 provides a schematic of a reaction to produce alternative compounds of the invention.

Figure 3 is a bar graph illustrating mean CAT expression for various compounds of the invention.

Figure 4 provides mean CAT expression for various compounds of the invention.

Description of Specific Embodiments

The present invention relates to non-viral, non-lipid compositions for gene delivery. The compositions contain a cationic amino sugar derivative that associates with negatively-charged nucleic acids via electrostatic interactions.

The cationic amino sugar derivatives may be used to deliver DNA for a variety of purposes. In one preferred embodiment they are used to deliver DNA prophylactically to generate an immune response against a desired antigen. Similarly, the polymers may be used to deliver nucleic acids as a therapeutic vaccine. By therapeutic vaccine, is meant the treatment of an existing condition by stimulation of an immune response. Examples of conditions that can be treated using the methods of the invention include but are not limited to cancer, autoimmune diseases, infectious diseases, and allergies.

Cationic Amino Sugar Derivatives

The cationic amino sugar derivatives can be prepared from the desired sugars and cationic groups using standard organic synthesis techniques. In some preferred embodiments, aminoglycosides are used, which can be prepared using standard

synthetic techniques and in the case of the antibiotics are available from common commercial sources such as Fluka (Milwaukee, WI), Sigma (St Louis, MO), and Aldrich (Milwaukee, WI). Compounds such as amikacin, arbekacin, deoxydihydrostreptomycin, destomycin A, dibekacin, dihydrostreptomycin, genticin, gentamicin, hygromycin B, isepamycin, kanamycin A, kanamycin B, kanamycin C, micronomycin, paromomycin, ribostamycin, streptomycin, streptonicozid, and tobramycinbutirosin, neomycin, and paromomycin, as well as methods of making them, are well known to those skilled in the art.

In some embodiments, it may be desirable to adjust the distance between the cationic domain and the sugar to modulate the solubility and/or for target specificity of the molecule. This can be achieved by including appropriate spacer groups between the sugar and the cationic domain. For instance, small amino acids such as alanine can be inserted between the sugar and the cationic group to adjust the length of the molecule. Means for preparing such compounds are well known to those skilled in the art.

Nucleic Acids

The nucleic acids which are useful in the present invention are typically nucleotide polymers having from 10 to 100,000 nucleotide residues, typically plasamid DNA. Typically, the nucleic acids are to be administered to a subject for the purpose of inducing an immune response, replacing or enhancing the expression of a cellular protein and may also encode an RNA molecule, *e.g.* antisense RNA or ribozyme, which will inhibit an undesired cellular activity, *e.g.* in a virus-infected cell or tumor cell. Additionally, the nucleic acid can carry a label (*e.g.*, radioactive label, fluorescent label or colorimetric label) for the purpose of providing clinical diagnosis relating to the presence or absence of complementary nucleic acids. Accordingly, the nucleic acids, or nucleotide polymers, can be polymers of nucleic acids including genomic DNA, cDNA, mRNA or oligonucleotides containing nucleic acid analogs, for example, the antisense derivatives described in a review by Stein, *et al.*, *Science* **261**:1004-1011 (1993) and in U.S. Patent Nos. 5,264,423 and 5,276,019, the disclosures of which are incorporated herein by reference. Still further, the nucleic acids may encode transcriptional and translational regulatory sequences including promoter sequences and enhancer sequences.

The nucleic acids can be single-stranded DNA or RNA, or double-stranded DNA or DNA-RNA hybrids. Examples of double-stranded DNA include structural genes including control and termination regions.

Single-stranded nucleic acids include antisense oligonucleotides (complementary to DNA or RNA), ribozymes and triplex-forming oligonucleotides. In order to increase stability, some single-stranded nucleic acids will preferably have some or all of the nucleotide linkages substituted with stable, non-phosphodiester linkages, including, for example, phosphorothioate, phosphorodithioate, phosphoroselenate, or O-alkyl phosphotriester linkages.

The nucleic acid may be in any physical form, e.g., linear, circular or supercoiled; single-stranded, double-, triple-, or quadruple-stranded; and further including those having naturally occurring nitrogenous bases and phosphodiester linkages as well as non-naturally occurring bases and linkages, e.g. for stabilization purposes. Preferably it is in the form of supercoiled plasmid DNA. Plasmid DNA is conveniently used for DNA transfections since there are no size constraints on the DNA sequences that may be included, and it can be produced in large quantity by growing and purifying it from bacterial cells.

The nucleic acids used in the present invention will also include those nucleic acids in which modifications have been made in one or more sugar moieties and/or in one or more of the pyrimidine or purine bases. Examples of sugar modifications include replacement of one or more hydroxyl groups with halogens, alkyl groups, amines, azido groups or functionalized as ethers or esters. Additionally, the entire sugar may be replaced with sterically and electronically similar structures, including aza-sugars and carbocyclic sugar analogs. Modifications in the purine or pyrimidine base moiety include, for example, alkylated purines and pyrimidines, acylated purines or pyrimidines, or other heterocyclic substitutes known to those of skill in the art.

Multiple genetic sequences can also be used in the present methods. Thus, the sequences for different proteins may be located on the same or separate plasmids. The gene of interest will be linked to appropriate regulatory elements to provide constitutive or inducible expression and/or tissue specific expression. Additional elements such as antibiotic-sensitive or nutrient-sensitive regions, may be included as required.

Non-coding sequences may also be present for various purposes including, for example, introns or regulatory elements to achieve appropriate expression or replication in host cells, or to provide convenient cloning sites.

The nucleic acids used in the present invention can be isolated from natural sources, obtained from such sources as ATCC or GenBank libraries or prepared by synthetic methods.

Synthetic nucleic acids can be prepared by a variety of solution or solid phase methods. Generally, solid phase synthesis is preferred. Detailed descriptions of the procedures for solid phase synthesis of nucleic acids by phosphite-triester, phosphotriester, and H-phosphonate chemistries are widely available. See, for example, Itakura, U.S. Pat. No. 4,401,796; Caruthers, *et al.*, U.S. Pat. Nos. 4,458,066 and 4,500,707; Beaucage, *et al., Tetrahedron Lett.*, **22**:1859-1862 (1981); Matteucci, *et al., J. Am. Chem. Soc.*, **103**:3185-3191 (1981); Caruthers, *et al., Genetic Engineering,* **4**:1-17 (1982); Jones, chapter 2, Atkinson, *et al.*, chapter 3, and Sproat, *et al.*, chapter 4, in *Oligonucleotide Synthesis: A Practical Approach*, Gait (ed.), IRL Press, Washington D.C. (1984); Froehler, *et al., Tetrahedron Lett.*, **27**:469-472 (1986); Froehler, *et al., Nucleic Acids Res.*, **14**:5399-5407 (1986); Sinha, *et al. Tetrahedron Lett.*, **24**:5843-5846 (1983); and Sinha, *et al., Nucl. Acids Res.*, **12**:4539-4557 (1984) which are incorporated herein by reference.

The nucleic acid may encode any desired therapeutic gene or, in preferred embodiments, may encode any antigen for which it is desirable to elicit an immune response. Genes of interest for vaccination purposes include any gene for which genetic immunization is useful, including genes encoding an antigen from an infectious viral, prokaryotic or eukaryotic pathogen. Many such antigens are known and include, for example, antigens for malaria, influenza, bovine herpesvirus, hepatitis B, hepatitis C, HIV, rabies, herpes simplex virus, papillomavirus, lymphocytic choriomeningitis virus, flavivirus, leishmaniasis, M. tuberculosis, M. pulmonis, and schistosomiasis. See Ulmer, J.B., et al., (1996) Curr. Opin. Immunol. 8:531-536. Genes encoding tumor-associated or tumor-specific antigens are also known and may be used in a therapeutic cancer vaccine.

For autoimmune disorders, T-cell receptor genes, derived from autoreactive T-cells, may be used to stimulate an immune response and attenuation of the

disease. See, e.g., Waisman, A., et al. (1996) Nat. Med. 2:899-905. Vaccination with DNA encoding allergens may be useful in the treatment of allergies. See, e.g., Hsu, C-H, et al. (1996) Nat. Med. 2:540-544; Raz, E., et al. (1996) Proc. Natl. Acad. Sci. USA 93:5141-5145.

Additional genes may also be delivered to further stimulate or modulate the immune response. See Hengge, U., et al. (1995) Nat. Genet. 10:161-166; Ziang, Z and Ertl H.C. (1995) Immunity 2:129-135; Conry, R.M., et al. (1996) Gene Ther. 3:67-74. Examples of useful cytokine genes include IL2, IL12 and IFN-gamma for stimulating a TH1 response; IL4, and IL10 for stimulating a TH2 response, and GM-CSF for stimulating effects on other cells of the immune system. Other genes such as B7-1 and B7-2 also find use in enhancing the immune response.

The genes to be expressed will be delivered with regulatory control elements known in the art, such as a promoter, enhancer and polyadenylation signal. The promoter may be a constitutive promoter, or an inducible promoter, and may or may not be a tissue-specific promoter. Examples of useful promoters include promoters from cytomegalovirus (CMV), Rous sarcoma virus (RSV), Simian Virus 40 (SV40), Mouse mammary tumor virus (MMTV), Maloney murine leukemia virus long terminal repeat (MMLV LTR), human immunodeficiency virus long terminal repeat (HIV LTR), Herpes Simplex virus thymidine kinase (HSV TK), human actin, human myosin, human hemoglobin, human muscle creatnine, and human metallothionein.

Enhancers useful for increasing or modulating gene expression include, for example, CMV, immunoglobulin, IL2 and beta-globin enhancers. Additional regulatory sequences are known in the art and may be included to improve gene expression. For example, a Kozak consensus sequence may be included to improve translation efficiency. See Francis et al. (1992) Biochim. Biophys. Acta 1130:29; Kozak, M. (1989) Mol. Cell. Biol. 9:5073. Improved expression may also be obtained by inclusion of an intron, such as the pre-proinsulin intron, and the like. In addition, the gene coding sequence may be optimized for expression by selection of codons that are used most efficiently in the target cell.

For stimulation of an immune response, it is also desirable to include immunostimulatory sequences in the nucleic acid vector. Enhanced immune

responsiveness is seen with vectors containing GC-rich bacterial DNA, in particular, a motif containing unmethylated purine-purine-C-G-pyrimidine-pyrimidine sequences. WO 97/28259; Krieg, A.M., et al. (1995) Nature 374:546-549; Sato, Y., et al. (1996) 273:352-354.

Cationic Amino Sugar Derivative-Nucleic Acid Complexes

The cationic amino sugar derivatives of the present invention interact with a nucleic acid molecule through a charge interaction with the cationic group. When condensed to form a complex with the cationic amino sugar derivative the nucleic acid can form a stable complex of an appropriate size. For Intramuscular delivery, the appropriate size is from about 50 nm to about 500 nm, and typically less than about 200 nm. However, in some formulations particulates may not form, in which case size of a complex will not be measurable. In addition, the delivery system is capable of protecting the nucleic acid and maintaining reasonable stability in the biological environment. The formed complexes can then facilitate cellular uptake and ultimately gene expression. Furthermore, the formed complexes preferably dissociate in the cell to release the nucleic acid for expression. The delivery system compounds also have low toxicity and are biodegradeable. In addition, the cationic amino sugar derivatives may be modified to target certain cells by forming specifically binding molecules on the surface of the target cell.

The cationic amino sugar derivatives are complexed with the nucleic acid component in different ratios depending on the target cell type, generally ranging from about 1:0.5 to about 1:32 μg DNA:nmole cationic amino sugar derivative and preferably from about 1:1 to about 1:8 μg DNA:nmole cationic amino sugar derivative, expressed in terms of weight:weight ratios, preferred ranges are from about 1:0.5 to about 1:0.1. Additional parameters such as nucleic acid concentration, charge of the amino sugar compound, buffer type and concentration, etc., will have an effect on transfection efficiency, and can be optimized by routine experimentation by a person of ordinary skill in the art. Preferred conditions are described in the Examples that follow.

In some embodiments, static mixers are used to mix the nucleic acids and amino sugars of the invention, particularly where gentle and complete mixing is desired.

The term "static mixer" refers to any flow through device which provides enough contact time between two or more liquids to allow substantially complete mixing of the liquids. Typically, static mixers contain an internal helical structure which allows the liquids to come in contact in an opposing rotational flow and causes them to mix in a turbulent or laminar flow. Such mixers are described, for instance, in U.S. Patent No. 3,286,922.

A number of analytical methods are known for characterizing the complexes prepared according to the method of the invention. Visual inspection may provide initial information as to aggregation of the complexes. Spectrophotometric analysis may be used to measure the optical density, giving information as to the aggregated status of the complexes; surface charge may be determined by measuring zeta potential; agarose gel electrophoresis may be utilized to examine the amounts and physical condition of the polynucleotide molecules in the complexes; particle sizing may be performed using commercially available instruments; HPLC analysis will give additional information as to resulting component ratios; and dextrose or sucrose gradients may be used to analyze the composition and heterogeneity of complexes formed.

Contacting Cells With Cationic Amino Sugar Derivatives-Nucleic Acid Complexes

Following formation of the cationic amino sugar-nucleic acid complexes, the complexes can be contacted with the cells to be transfected. Contact between the cells and the cationic amino sugar-nucleic acid complexes, when carried out *in vitro*, will take place in a biologically compatible medium, preferably without serum. The amount of complexes can vary widely depending on the particular application, but is generally between about 0.5 μg DNA, preferably between about 1 and about 1.5 μg DNA per 500,000 cells in a 35 mm dish. Treatment of the cells with the cationic amino sugar-nucleic acid complexes will generally be carried out at physiological temperatures (about 37EC) for periods of time of from about 1 to 48 hours, preferably of from about 2 to 4 hours. For *in vitro* applications, the delivery of nucleic acids can be to any cell grown in culture, including primary cell and immortalized cell lines, whether of plant or animal origin, vertebrate or invertebrate, and of any tissue or type. In preferred embodiments, the cells will be animal cells, more preferably mammalian cells, and most preferably human cells.

Typical applications include using well known transfection procedures to provide intracellular delivery of DNA sequences which code for therapeutically useful polypeptides. However, the compositions can also be used for the delivery of the expressed gene product or protein itself. In this manner, therapy is provided for genetic diseases by supplying deficient or absent gene products (*i.e.*, for Duchenne's dystrophy, see Kunkel, *et al., Brit. Med. Bull.* **45(3):**630-643 (1989), and for cystic fibrosis, see Goodfellow, *Nature* **341:**102-103 (1989)). Other uses for the compositions of the present invention include introduction of antisense oligonucleotides in cells (see, Bennett, *et al., Mol. Pharm.* **41:**1023-1033 (1992)).

In one group of embodiments, the *in vivo* administration of the pharmaceutical compositions is carried out parenterally, *e.g.*, intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. More preferably, the pharmaceutical compositions are used for local delivery applications such as intra-articularly for the treatment of rheumatoid arthritis, osteoarthritis, or cartilege repair, or by intramuscular or subcutaneous injection for delivery to antigen-presenting cells for purposes of eliciting an immune response or for expression of therapeutic proteins to be secreted and active systemically. For example, see Stadler, *et al.*, U.S. Patent No. 5,286,634, which is incorporated herein by reference. Intracellular nucleic acid delivery has also been discussed in Straubringer, *et al.*, METHODS IN ENZYMOLOGY, Academic Press, New York. **101:**512-527 (1983); Mannino, *et al., Biotechniques* **6:**682-690 (1988); Nicolau, *et al., Crit. Rev. Ther. Drug Carrier Syst.* **6:**239-271 (1989), and Behr, *Acc. Chem. Res.* **26:**274-278 (1993).

In other embodiments, the pharmaceutical preparations described herein may be contacted with the target tissue by direct application of the preparation to the tissue. The application may be made by topical, "open" or "closed" procedures. By "topical", it is meant the direct application of the pharmaceutical preparation to a tissue exposed to the environment, such as the skin, oropharynx, external auditory canal, and the like. "Open" procedures are those procedures that include incising the skin of a patient and directly visualizing the underlying tissue to which the pharmaceutical preparations are applied. This is generally accomplished by a surgical procedure, such as a thoracotomy to access the lungs, abdominal laparotomy to access abdominal viscera, or

other direct surgical approach to the target tissue, for example, heart tissue and/or associated vasculature may be transfected with a gene of interest in conjunction with heart surgery, as an adjunct therapy. "Closed" procedures are invasive procedures in which the internal target tissues are not directly visualized, but accessed via inserting instruments through small wounds in the skin. For example, the preparations may be administered to the peritoneum by needle lavage or to the vasculature using a balloon catheter. Likewise, the pharmaceutical preparations may be administered to the meninges or spinal cord by infusion during a lumbar puncture followed by appropriate positioning of the patient as commonly practiced for spinal anesthesia or metrazamide imaging of the spinal cord. Alternatively, the preparations may be administered through endoscopic devices.

In yet other embodiments, the cationic amino sugar -nucleic acid complexes can be administered in an aerosol inhaled into the lungs. For a general review of applicable techniques, see U.S. Patent No. 5,641,662; Culver, HUMAN GENE THERAPY, MaryAnn Liebert, Inc., Publishers, New York. pp.70-71 (1994).

The methods of the present invention may be practiced in a variety of hosts. Preferred hosts include mammalian species, such as humans, non-human primates, dogs, cats, cattle, horses, sheep, and the like.

The transfection complexes of the present invention are particularly useful for generating an immune response when administered via intramuscular or dermal routes of administration. For dermal routes of administration, delivery may be by epidermal administration, subcutaneous or intradermal injection. Preferably delivery is by intramuscular or intradermal administration.

It may also be desirable to provide some irritant at the site of administration to attract antigen-presenting cells (APCs) to the site. For example, a mechanical irritant may be used, which employs several short tynes that irritate the skin and attract APCs. The transfection complexes may be transferred from the ends of the tynes. Alternatively, the irritant may be a chemical irritant, such as bupivicaine. US Patent No. 5,593,972.

The dose of nucleic acid delivered will depend on the prophylactic or therapeutic application. Dosages can be adjusted to achieve the desired level of transgene expression and/or level of immune response. Means to confirm the presence and quantity

of the encoded protein and/or mRNA levels are well known in the art, and include, for example, PCR, immunoassays, and immunohistochemistry. Methods of measuring the immune response are known in the art. In addition, animal models are available for determining the level of protection provided by a vaccination protocol using methods well known in the art.

Typically, the dose administered will be from about 0.05 to about 5 mg/kg for stimulation of an immune response, and may be as low as 0.01 mg/kg. The dosing regimen will typically that employed by known genetic vaccination and immunotherapy protocols, and may include priming doses, and may be followed by booster and/or maintenance doses of nucleic acids encoding the antigen of interest.

Furthermore, the pharmaceutical compositions may also include an adjuvant. As used here, a number of adjuvants are well known to one skilled in the art. Suitable adjuvants include incomplete Freund's adjuvant, alum, aluminum phosphate, aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-Lalanyl-D-isoglutaminyl-L,,alanine-2-(1'-2'-dipalmitoyl-sn,,glycero-3-hydroxyphosphoryloxy),,ethylamine (CGP 19835A, referred to as MTP-PE), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion. The effectiveness of an adjuvant may be determined by measuring the amount of antibodies directed against the immunogenic peptide.

The invention will be better understood in light of the following specific examples, which are merely illustrative and should not be construed as limiting the invention in any respect, as will be evident to those skilled in the art.

EXAMPLES

The examples and embodiments described herein are for illustrative purposes only, and various modifications will be apparent to those of skill in the art, the invention to be limited only by the scope of the appended claims. All publications, patents and patent applications cited herein are hereby incorporated by reference as if set

forth in their entirety herein.

Example 1: Synthesis of Cationic Galactose Derivative

A schematic of the reaction used to synthesis the compounds of the invention is shown in Figure 1.

To a solution of 1,2,3,4-Di-O-isopropyline-α-D-galactopyranose (12.5 g, 48.1 mmol), triphenylphosphine (13.9 g, 52.9 mmol), and phthalimide (7.86 g; 53.4 mmol) in dry THF (200 ml) was added dropwise to diethyl azoclicarboxalate (10.1 g, 5 8.2 mmol). The yellow solution was stirred at room temperature overnight. The solvent was removed under reduced pressure, and the residue was taken up to Et_2O /Hexanes (1:1, 100 mL) and stirred for 30 min. The mixture was filtered, the solid suspended in Et_2O /Hexanes (1:1, 100 mL) and stirred for 10 min, and then filtered again. This filtration and suspension procedure repeated 3 times. The combined filtrate was concentrated to dryness and residue oil was purified on a silica gel column (EtOAc/Hexanes, 0-10%) to afford the corresponding imide as a white solid (11.74 g, 62.7 %, mp 90-93°C). ^{1}H NMR ($CDCl_3$, 300 MHz): δ1.27 (s, 3H), 1.38 (s, 3H), 1.46 (s, 3H), 1.55 (s, 3H), 3.65 (d, J=13 Hz, 1H), 4.26 (m, 4H), 4.64 (m, 1H), 5.47 (d, J = 5 Hz, 1H), 7.71 (m, 2H), 7.85 (m, 2H). A mixture of the imide obtained above (5 g, 12.85 mmol), hydrazine hydrate (0.9 mL, 15.42 mmol) in EtOH (100 mL) was heated under reflux for 4h. After cooling to room temperature, the mixture was filtered and the white solid washed with EtOH (50 mL) then Et_2O (2 x 20 mL). The filtrate was concentrated to dryness again and the residue purified by column chromatography (SiO_2, EtOAc/Hexanes, 30-50%; EtOAc; MeOH/EtOAc, 5%). The amino galactopyranose **41-a** (2.9 g, 54.6 %, 2 steps) was obtained as a colorless oil. ^{1}H NMR ($CDCl_3$, 300 MHz): δ1.33 (s, 6H), 1.45 (s, 3H), 1.53 (s, 3H), 2.90 (m, 2H), 3.70 (m, 1H), 4.23 (dd, J = 8, 2 Hz, 1H), 4.32 (dd, J = 5, 2 Hz, 1H), 4.60 (dd, J = 8, 2 Hz, I H), 5.5 5 (d, J = 5 Hz, 1 H).

To a solution of β-N-Fmoc-alanine (4.18 g, 13.44 mmol), Et3N (2.18 mL, 15.68 mmol) in CH_2Cl_2 (60 mL) was added BOP (6.44 g, 14.56 mmol) and the mixture stirred at room temperature for 10 min. A solution of **41-a** (2.8 g, 11.20 mmol) in CH_2Cl_2 (20 mL) was added. A few drops Et_3N was added to adjusted the pH to higher than 8. After stirring at room temperature overnight, the solvent was evaporated under reduced

pressure. The residue was purified on a silica gel column (EtOAc/Hexanes 0-30%) to give the β-N-Fmoc-alanine amide (4.78 g, 77.2%) as a white solid, mp 101-104°C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ1.30 (s, 3H), 1.34 (s, 3H), 1.46 (s, 3H), 1.47 (s, 3H), 2.35 (m, 2H), 3.15 (m, 1H), 3.35 (m, 1H), 3.60 (m, 1H), 3.75 (m, 1H), 4.00 (m, 1H), 4.20 (m, 2H), 4.29 (m, 1H), 4.42 (m, 2H), 4.60 (m, 1H), 5.39 (m, 1H), 5.89 (m, 2H), 7.31 (m, 2H), 7.40 (m, 2H), 7.76 (d, J = 7 Hz, 2H). A solution of above β-N-Fmoc-alanine amide (2.95 g, 5.33 mmol) in piperidine (15 mL) was stirred at room temperature for 2 h and diluted with H_20 (100 mL). The resulting white precipitate was filtered and washed with H_20 (2 x 10 mL). The filtrate was extracted with EtOAc (10 x 50 mL) until no any more product coming out determined by TLC. The EtOAc solution was dried over $MgSO_4$ and evaporated to dryness giving the deprotected product **41-b** (1.6 g, 90.8%) as a yellowish oil. ^{1}H NMR ($CDCl_3$, 300 MHz): δ1.31 (s, IH), 1.33 (s, 3H), 1.42 (s, 3H), 1.48 (s, 3H), 2.33 (t, J = 6 Hz, 2H), 3.01 (t, J = 6 Hz, 2H), 3.20 (m, 1H), 3.70 (m, I H), 3.92 (m, 1H), 4.20 (m, 1H), 4.30 (m, 1H), 4.59 (m, 1H), 5.50 (d, J = 5 Hz, 1 H).

To a solution of tetra-Boc-5-carboxyspermine (2.11 g, 3.26 mmol), Et_3N (0.61 mL, 4.35 mmol) in CH_2Cl_2 (20 mL) was added BOP (1.73 g, 3.92 mmol) and the mixture stirred at room temperature for 10 min. A solution of **41-b** (0.9 g, 2.72 mmol) in CH_2Cl_2 (30 mL), washed with H_2O (2 x 20 mLt), NaCl (20 mL) and dried. The solvent was evaporated and the residue was purified on a silica fel column (EtOAc/Hexanes 0 -30 -80%) giving the coupling product **41-c** (0.83 g, 31.8 %). ^{1}H NMR ($CDCl_3$, 300 MHz): δ1.26 (m, 12H), 1.43 (m, 40H), 1.65 (m, 4H), 2.35 (m, 2H), 3.00-3.70 (m, 14H0, 4.02-4.25 (m, 3H), 4.35 (m, 1H), 4.61 (m, 1H0, 5.55 (m, 1H).

A mixture of above coupling product **41-c** (790 mg, 0.824 mmol) and a solution of HCl in Dioxane (4 M, 15 mL) was stirred at room temperature for 5 h. The solvent was evaporated under reduced pressure, and the residue was washed with acetone (2 x 5 mL) and Et2O (2 x 5 mL). The obtained solid was dissolved in a mixture of IN HCl/TBF (1:1, 10 mL) and stirred at room temperature for 2 h. After removing the solvent under reduced pressure, the residue charged on an activated charcoal column and eluted with MeOH. Concentration gave the product **MBD-41** (550 mg, 85%) as a yellowish foaming, very hygroscopic solid. ^{1}H NMR (D_2O, 300 MHz): δ .50-2.15 (m,

8H), 2.42 (m, 2H), 3.03 (m, 8H), 3.10-4.03 (m, 12H). Anal. Calcd for $C_{20}H_{41}N_6O_7$. 4HCl. 1/2H_2O. 2CH_3OH. 1.2HCl: C 35.16, H 7.26, N 11.19. Found: C 35.09, H 7.06, N 11.24.

Figure 2 provides a schematic for synthesis of alternative compounds of the invention using a synthetic scheme similar to the one described above. In this scheme, the free hydroxy is activated by reaction with toluene sulfonyl chloride in pyridine to produce the corresponding tosyl ester, which is subsequently converted to the corresponding amine by reaction with methanolic ammonia at 120°C.

The amine can be reacted with the N-hydroxysuccinimidyl ester of tetra-Boc-5-carboxyspermine. The product of this reaction can be deprotected in HCL and dioxane to provide MBD-55, a compound of the invention.

Example 2: Contacting Cell with Cationic Amino Sugar-Nucleic Acid Complex

Each formulation of complex was prepared by mixing equal volumes of the DNA solution and the aminoglycoside solution with the appropriate buffer. The aminoglycoside solution was hand pipetted into a vortexing DNA solution. For example, 0.66 mg/ml of aminoglycoside solution was suspended in 5 % dextrose in water (w/v) (D5W) saline buffer to a volume of 0.5 ml. This solution was hand pipetted into a 5 ml solution of 2.0 mg/ml DNA solution in D5W, saline buffer. The final solution consisting of 1.0 mg/ml DNA, 0.33 mg/ml aminoglycoside in D5W, saline buffer (total volume 1.0 ml) was vortexed at #3 on a vortex Genie II. The DNA used was an expression plasmid containing the CAT reporter gene under the control of the CMV promoter.

Then 50 µl doses of complexes were administered intramuscularly to right anterior tibialis muscles of ICR(CD-1) mice. After 24 hours, the muscle samples were harvested and tested for CAT expression by ELISA. Mean CAT expression results for two trials, expressed as pg CAT/mg soluble protein, are shown in Figure 3 and Figure 4 for compounds of the invention. Four separate trials are summarized in Table 1, in which expression results are compared to naked DNA. Formulations were prepared and assayed as described above.

The compound shown in Figure 1 (MBD-41) was also assayed as described above, in a formulation containing MBD-41 at 0.25 mg/ml, NaCl at 9 mg/ml and CAT plasmid DNA at 0.2 mg/ml. 50 µl was delivered to ten mice and compared to

naked DNA at the same DNA dose. The results showed expression in 10/10 mice with a mean of 45254 ± 52689 pg CAT/mg soluble protein, compared to naked DNA CAT expression at 37428 ± 38608 pg CAT/mg soluble protein.

TABLE 1

CAT Expression (pg/mg) X-times higher than naked DNA control

Formulation	**DNA conc. (mg/ml)**	**Average**	**Trial 1**	**Trial 2**	**Trial 3**	**Trial 4**
Naked DNA	1.0		24600 ± 28066	6607 ± 5202	13427 ± 7107	29981 ± 41472
Paromomycin	1.0	9.2x (2/2)		17.2x		1.2x
Butirosin	1.0	5.2x (2/3)	3.1x	7.3x		Ø
Neomycin	1.0	4.4x (2/3)	Ø	7.3x		1.4x
Streptomycin	1.0	3.7x (3/3)	1.9x	7.9x		1.3x
Eledoisin	1.0	1.5x (1/2)			1.5x	Ø

Ø indicates that expression was below that of naked DNA control.

WHAT IS CLAIMED IS:

1. A method of introducing a DNA molecule into a cell of a mammal, the method comprising contacting a cell with a complex comprising the nucleic acid molecule and a cationic amino sugar derivative.

2. The method of claim 1, wherein the cationic amino sugar derivative is an aminoglycoside.

3. The method of claim 1, wherein the cationic amino sugar derivative is an antibiotic.

4. The method of claim 3, wherein the cationic amino sugar derivative is monosaccharide.

5. The method of claim 4, wherein the cationic amino sugar derivative is selected from the group consisting of amiprilose and streptidine.

6. The method of claim 3, wherein the cationic amino sugar derivative is disaccharide.

7. The method of claim 6, wherein the cationic amino sugar derivative is selected from the group consisting of fortimicins, neamine, sisomicin, streptobiosamine,

8. The method of claim 3, wherein the cationic amino sugar derivative is a trisaccharide.

9. The method of claim 8, wherein the cationic amino sugar derivative is selected from the group consisting of amikacin, arbekacin, deoxydihydrostreptomycin, destomycin A, dibekacin, dihydrostreptomycin, genticin, gentamicin, hygromycin B, isepamycin, kanamycin A, kanamycin B, kanamycin C, micronomicin, paromomycin, ribostamycin, streptomycin, streptonicozid, and tobramycin.

10. The method of claim 1, where in the cationic amino sugar derivative comprises a spermine moiety.

11. The method of claim 1, wherein the complex is delivered intramuscularly.

12. The method of claim 1, wherein the complex is delivered intradermally.

13. The method of claim 1, wherein the complex is delivered intraperitoneally.

14. The method of claim 1, wherein the complex is delivered intratracheally.

15. The method of claim 1, wherein the complex is delivered intra-articularly.

16. The method of claim 14, wherein the DNA molecule comprises an expression cassette.

17. The method of claim 16, wherein the expression cassette encodes a therapeutic protein.

18. The method of claim 16, wherein the expression cassette encodes a protein that induces an immune response in the mammal.

19. A pharmaceutical composition comprising a pharmaceutically acceptable excipient and a complex comprising the nucleic acid molecule and a cationic amino sugar derivative.

20. The composition of claim 19, wherein the cationic amino sugar derivative is an aminoglycoside.

21. The composition of claim 19, wherein the cationic amino sugar derivative is an antibiotic.

22. The composition of claim 19, wherein the cationic amino sugar derivative is monosaccharide.

23. The composition of claim 22, wherein the cationic amino sugar derivative is selected from the group consisting of amiprilose and streptidine.

24. The composition of claim 19, wherein the cationic amino sugar derivative is disaccharide.

25. The composition of claim 24, wherein the cationic amino sugar derivative is selected from the group consisting of fortimicins, neamine, sisomicin, streptobiosamine,

26. The composition of claim 19, wherein the cationic amino sugar derivative is a trisaccharide.

27. The composition of claim 26, wherein the cationic amino sugar derivative is selected from the group consisting of amikacin, arbekacin, deoxydihydrostreptomycin, destomycin A, dibekacin, dihydrostreptomycin, genticin, gentamicin, hygromycin B, isepamycin, kanamycin A, kanamycin B, kanamycin C, micronomicin, paromomycin, ribostamycin, streptomycin, streptonicozid, and tobramycin.

28. The composition of claim 19, where in the cationic amino sugar derivative comprises a spermine moiety.

29. The composition of claim 28, wherein the DNA molecule comprises an expression cassette.

30. The composition of claim 29, wherein the expression cassette encodes a therapeutic protein.

31. The composition of claim 29, wherein the expression cassette encodes a protein that induces a protective immune response in the mammal.

Synthesis of MBD-41

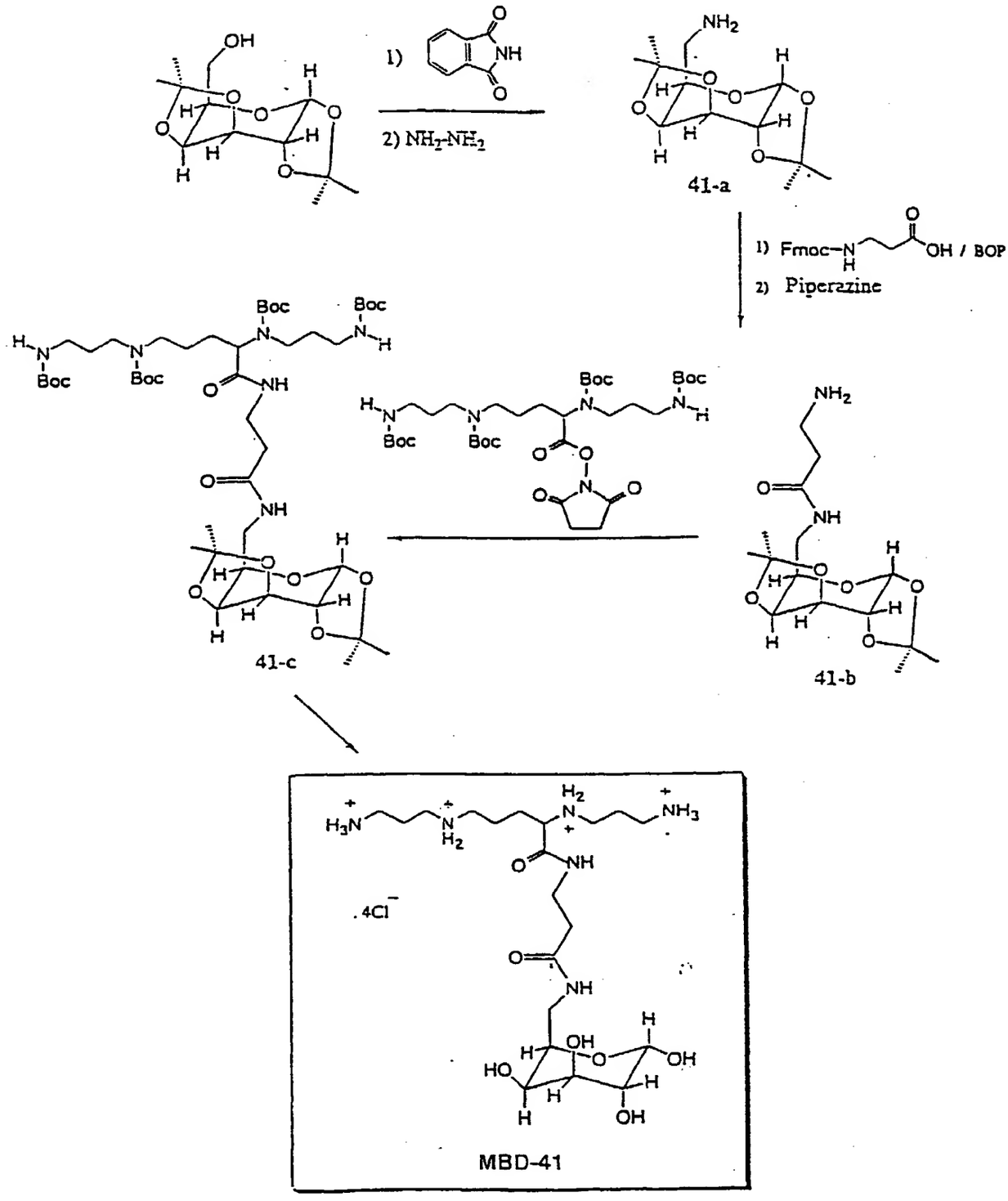

FIGURE 1

Synthesis of MBD-55

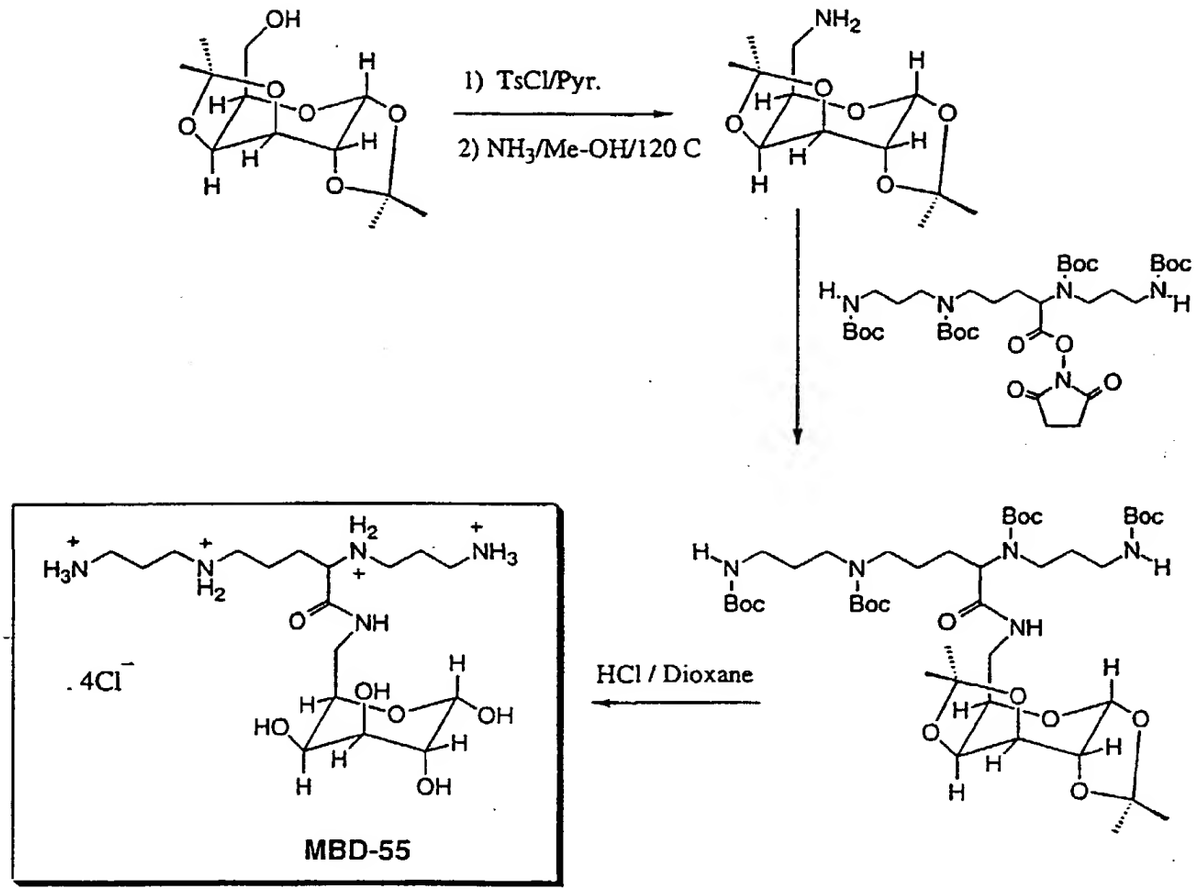

Figure 2.

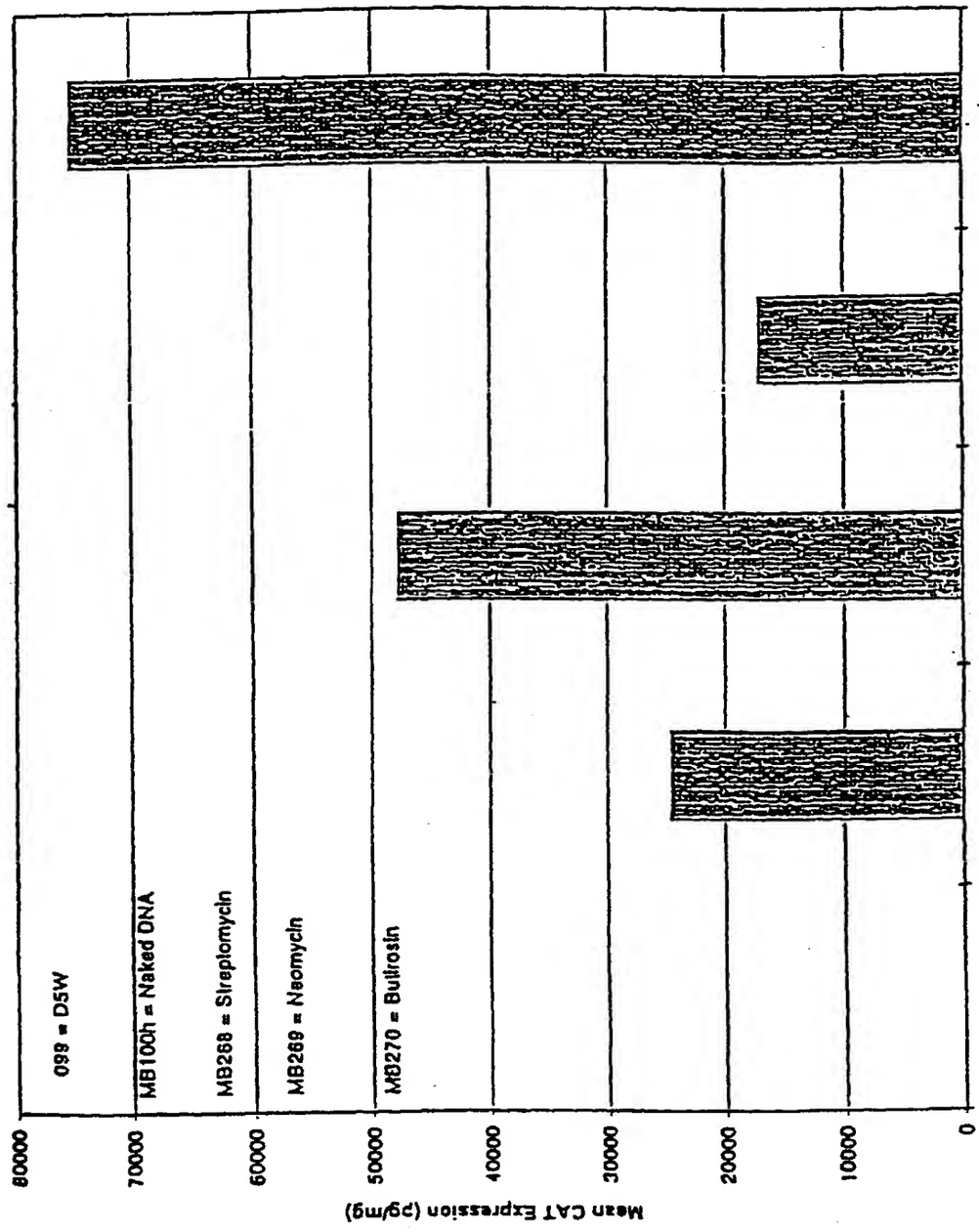
099 = D5W
MB100h = Naked DNA
MB268 = Streptomycin
MB269 = Neomycin
MB270 = Bulirosin
80000
70000
60000
50000
40000
30000
20000
10000
0
Mean CAT Expression (pg/mg)
99
MD100h-4119
MD260-4119
MD269-4119
MD270-4119
Formulation

Fig. 3

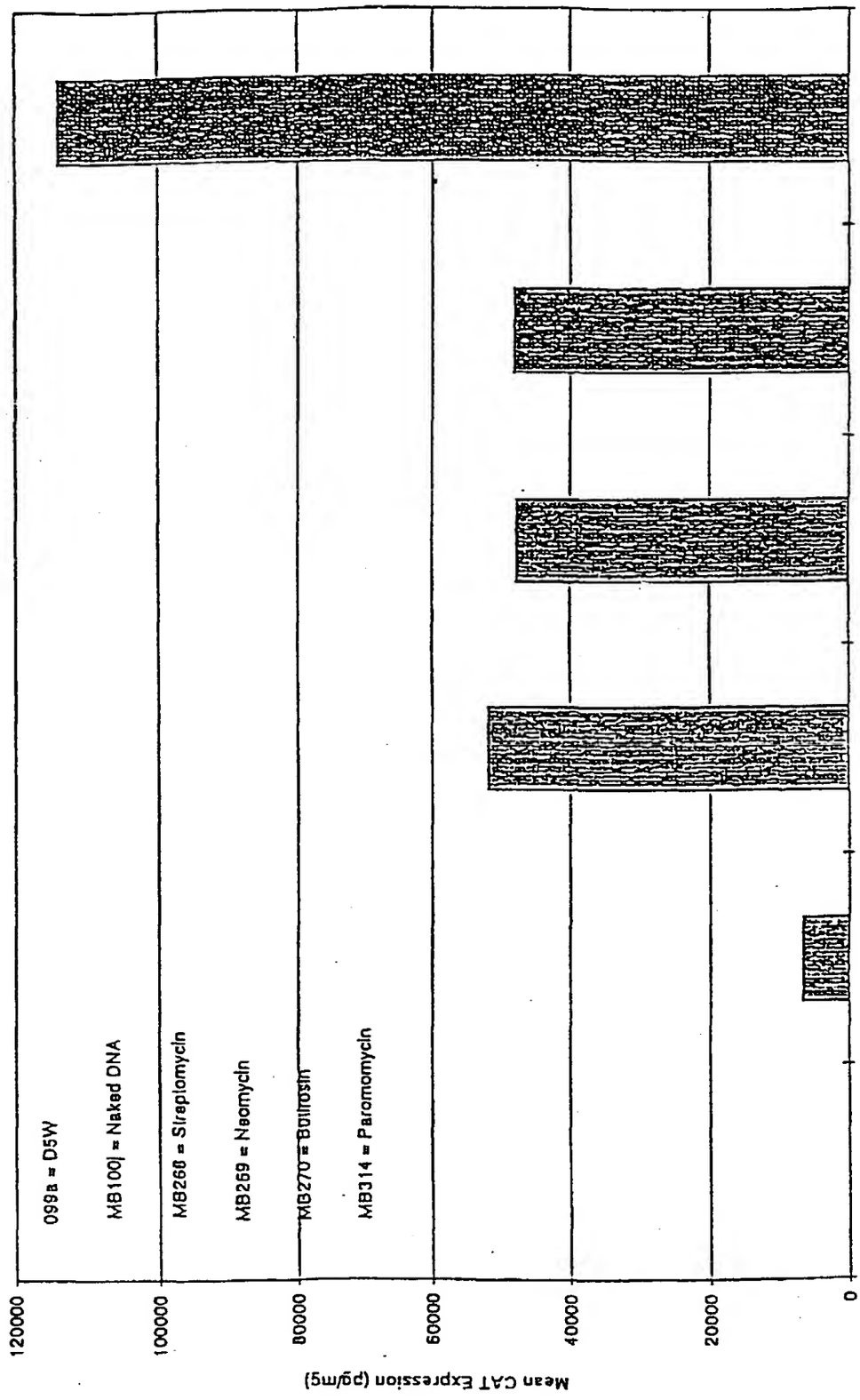
099a = D5W
MB100j = Naked DNA
MB260 = Streptomycin
MB269 = Neomycin
MB270 = Bulirosin
MB314 = Paromomycin
Mean CAT Expression (pg/mg)
120000
100000
80000
60000
40000
20000
0
099a
MB100j
MB260
MB269
MB270
MB314

Fig. 4